J. Clin. Chem. Clin. Biochem.
Vol. 20, 1982, pp. 9–13

The Effect of Storage at – 80 °C on the Activities of Cytoplasmic, Mitochondrial and Microsomal Enzymes in Rat Liver[1])

By *Christiane Bode* and *A. Meinel*[2])

Zentrum für Innere Medizin, Philipps-Universität Marburg/Lahn, Federal Republic of Germany

(Received April 6/August 14, 1981)

Summary: The effect of storage at – 80 °C for 1–28 days on the activity of 12 enzymes in intact liver tissue, liver extract and isolated hepatic microsomes was investigated. To find optimal conditions for tissue homogenization for this study the effects of three types of homogenization on the activity of 10 enzymes from different cell compartments were compared. The activities of glucokinase and phosphofructokinase decreased markedly during storage of both supernatant and liver tissue. Storage of liver tissue increased the activity of mitochondrial enzymes or isoenzymes. While this effect can be explained by additional disintegration of liver tissue caused by freezing and thawing for enzymes like glutamate dehydrogenase, other mechanisms may be involved in the prolonged increase observed in the activity of citrate synthase and xanthine oxidase during storage. The activity of a number of enzymes from the cytosol, mitochondria and microsomes decreased more markedly in the stored liver samples than in the stored supernatant or in the stored microsomal pellet.

Cytochrome P 450 content remained stable throughout the whole storage period in both intact liver tissue and isolated microsomes.

Einfluß einer Lagerung bei – 80 °C auf die Aktivität cytoplasmatischer, mitochondrialer und mikrosomaler Enzyme in der Rattenleber

Zusammenfassung: Es wurde der Einfluß der Lagerung bei – 80 °C auf die Aktivität von 12 Enzymen in intaktem Lebergewebe, dem Überstand eines Leberhomogenates und isolierten Lebermikrosomen über einen Zeitraum von 1–28 Tagen untersucht. Um optimale Bedingungen für den Gewebsaufschluß zu ermitteln, wurde zunächst der Effekt von 3 Homogenisationsverfahren auf die Aktivität von 10 Enzymen verschiedener zellulärer Lokalisation geprüft. Als schonendstes und damit für die geplante Untersuchung geeignetstes Verfahren erwies sich ein Gewebsaufschluß mit einem Homogenisator nach *Potter & Elvehjem.* Während der Lagerung nahm die Aktivität der Glucokinase und Phosphofructokinase sowohl im Überstand als auch in den Lebergewebeproben deutlich ab. Die Lagerung von Lebergewebe erhöhte die Aktivität mitochondrialer Enzyme bzw. Isoenzyme z.T. deutlich. Während dieser Effekt für Enzyme wie die Glutamatdehydrogenase auf einen zusätzlichen Gewebsaufschluß durch Frieren und Tauen erklärt werden kann, ist anzunehmen, daß für einen langsamen, kontinuierlichen Anstieg der Aktivität der Citratsynthase und Xanthinoxidase während der Lagerung von Lebergewebe andere Mechanismen mitverantwortlich sind. Die Aktivität mehrerer cytoplasmatischer, mitochondrialer und mikrosomaler Enzyme nahm im gelagerten Lebergewebe stärker ab als in dem tiefgefrorenen Überstand bzw. in isolierten Mikrosomen. Der Gehalt an Cytochrom P 450 blieb während der 4-wöchigen Lagerung sowohl im Lebergewebe als auch in isolierten Mikrosomen unverändert.

[1]) This work contains part of the dissertation of *Andreas Meinel*, FB Humanmedizin, Universität Marburg. Parts were presented at the 2nd Symposium on Experimental and Clinical Hepatology, Marburg, October 1979.

[2]) Present adress: Medizinische Klinik, Stadtkrankenhaus, D-3500 Kassel, Federal Republic of Germany.

0340-076X/82/0020-0009$02.00

Introduction

The effect of storage on the activity of single enzymes (1, 2, 3) and groups of enzymes (3, 4, 5) was the subject of earlier investigations, using intact liver tissue. These investigations were mainly performed at 0–4 °C (4, 5) or at – 20 °C (1, 5, 6), and, with one exception (6), the stability was tested only for short periods of time. Concerning enzymes in supernatants of liver homogenates, it has been stated that they cannot be stored for any appreciable length of time even at – 20 °C (5).

The aim of the present study was to investigate the effect of storage at – 80 °C on the activity of 12 enzymes in rat liver tissue, in supernatant of a liver homogenate and in isolated microsomes from the same liver lobe. The enzymes were selected partly for their relevance to other studies in this laboratory. For many enzymes the type of tissue homogenization distinctly influences the activity determined *in vitro* (3, 4). To define more clearly the optimal conditions for the present study, the effect of three homogenization procedures on the activity of 10 of the enzymes localized in the cytosol and/or mitochondria was compared prior to the storage experiments.

Material and Methods

Male Wistar rats (250–280 g body weight) from Ivanovas (Kißlegg, FRG) were used. The animals were allowed free access to a commercial standard diet (Altromin, Lage, FRG) and water. The food was withdrawn 12 h before the liver was removed under slight ether anesthesia between 9.00–1.00 a.m. Only tissue from the same major (right) liver lobe was used for all studies.

Study I

Preparation of tissue

Samples of about 1 g of liver were cut into pieces in the fivefold volume of KCl-solution (11.5 g/l). All procedures were carried out at 0–4 °C except where otherwise stated. Three types of tissue preparation were compared (3):

a) Homogenization by means of a *Potter-Elvehjem* homogenizer (teflon/glass; clearance 0.1 mm). To ensure constant conditions a drilling machine (type SB2E-600, AEG, FRG) with electronically regulated revolutions per minute was used. The samples were homogenized in ice 4 times for 30 s using a speed of 690 min^{-1} with one minute interruptions for cooling.

b) Homogenization with a blade homogenizer (Ultra-Turrax, Janke u. Kunkel, Staufen/Br., FRG). The homogenization was performed 4 times for 15 s (speed 15,000 min^{-1}) with one minute interruptions using an ice-salt-mixture (– 8 °C to – 10 °C) for cooling.

c) Combined homogenization by means of a *Potter-Elvehjem* homogenizer as described in section (a) and an additional homogenization using the Ultra-Turrax for 15 seconds. Following centrifugation at 100 000 *g* for 60 min (Spinco ultra centrifuge, Beckman Instruments) the supernatants of homogenates a)–c) were used immediately for the enzyme assays.

Study II

Effect of storage on enzyme activities

About 2 g of liver were taken for immediate homogenization using the *Potter-Elvehjem* homogenizer (see procedure a), study I). Four additional samples of about 0.6 g were stored at – 80 °C in sealed plastic tubes. Microsomes were isolated from the homogenate by fractionated centrifugation according to *Kato & Gilette* (7). The microsomal pellet (5 tubes) was stratified with the 9-fold volume of ice-cold KCl-solution (11.5 g/l). Four of the tubes were stored at – 80 °C, one was rehomogenized (*Potter-Elvehjem*-homogenizer, 2 × 15 s, 150 min^{-1}) for immediate assay.

Following centrifugation at 100 000 *g* the supernatant was divided into 5 equal volumes; again four of them were stored in sealed plastic tubes at – 80 °C, and one part was taken for immediate assay.

The stored samples were homogenized after 3, 7, 14 and 28 days respectively, using the procedures mentioned above. The enzyme activities were determined in both the stored supernatant (or isolated microsomes) and in the supernatant (isolated microsomes) of the stored samples of the same time intervals (3, 7, 14 and 28 days).

Enzyme Assays

Glucokinase (ATP: *D*-glucose 6-phosphotransferase; EC 2.7.1.2), glucose-6-phosphate dehydrogenase (*D*-glucose-6-phosphate: NADP 1-oxidoreductase; EC 1.1.1.49), fructose-bisphosphatase (*D*-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11), 6-phosphofructokinase (ATP: *D*-fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11), glutamate dehydrogenase (*L*-glutamate: NAD (P) oxidoreductase (deaminating); EC 1.4.1.3) were assayed as described by *Bücher* et al. (8).

L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1), *L*-alanine: 2-oxoglutarate aminotransferase (EC 2.6.1.2), xanthine oxidase (xanthine: oxygen oxidoreductase; EC 1.2.3.2), ornithine carbamoyl transferase (carbamoylphosphate: *L*-ornithine carbamoyltransferase; EC 2.1.3.3) and glucose-6-phosphatase (*D*-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) were assayed as described by *Bergmeyer* (9).

Citrate synthase (citrate oxaloacetate lyase; EC 4.1.3.7) was determined according to *Wieland* (10), and

Arginase (*L*-arginine ureohydrolase; EC 3.5.3.1) according to *Greenberg* (11).

Cytochrome c (b 5) oxidoreductase (EC 1.6.2. a) was determined with NAD and NADP as coenzyme by the method of *Mayer* et al. (12) and

Cytochrome P 450 according to *McLean* and *Day* (13) using a Beckman Acta V double beam spectrophotometer.

All the enzyme assays were carried out at 25 °C. The temperature in the cuvettes was controlled using a thermometer at intervals of about 1 h. The activities are expressed as µmol of substrate transformed per minute per gram fresh weight of tissue. Protein was determined by the Biuret method using KCN to eliminate lipid turbidity (14).

Results

Homogenization of liver tissue

The results obtained by comparing the 3 homogenization procedures are summarized in table 1. Using the *Potter-Elvehjem* homogenizer alone maximal activities were obtained in 6 out of 10 enzymes tested. Incomplete

Tab. 1. Effect of the type of homogenization on the activity of 10 enzymes in the 100 000 *g* supernatant of rat liver. Mean value of maximal activity = 1.00. Mean values of 6 assays of one liver. The coefficient of variation ranged between 2% and 7%.

Enzyme	Type of homogenization		
	I *Potter-Elvehjem* (4 × 30 s)	II Ultra-Turrax (4 × 15 s)	III = I + 15 s Ultra-Turrax
	Fraction of maximal activity		
Glutamate dehydrogenase	0.20	0.80	1.00
Citrate synthase	0.50	0.90	1.00
L-Aspartate: 2 oxoglutarate aminotransferase	0.75	1.00	1.00
L-Alanine: 2 oxoglutarate aminotransferase	1.00	0.80	0.60
Glucose-6-phosphate dehydrogenase	1.00	0.95	0.70
Fructose-6-phosphate-kinase	0.85	0.60	1.00
Hexokinase	1.00	0.70	0.75
Glucokinase	1.00	1.00	1.00
D-Fructose-bis-phosphatase	1.00	0.75	0.50
Ornithine carbamoyl transferase	1.00	0.20	0.20
Protein (mg/g)	59	162	141

extraction with this procedure was obtained for the three enzymes which are partially or totally localized in the mitochondria. In spite of careful cooling of the homogenate the Ultra-Turrax homogenization resulted in marked loss in the activity of several cytoplasmic enzymes. On the other hand with this type of homogenization maximal or near maximal activities were found for the soluble mitochondrial enzymes (glutamate dehydrogenase, citrate synthase, aspartate: 2-oxoglutarate aminotransferase). Maximal activities for the latter enzymes were also achieved by the combined method (tab. 1, III). However, even the short homogenization (15 seconds) with the Ultra-Turrax resulted in a pronounced fall in the activity of several enzymes of the cytosol.

Effect of storage at – 80 °C on liver enzyme activities

Stored supernatant

Storage of the 100 000 *g* supernatant lead to a distinct loss of the activity of glucokinase and 6-phosphofructokinase (fig. 1) while the activity of all other enzymes remained unchanged with the exception of xanthine oxidase, the activity of which exhibited a two-fold increase within 2 weeks (fig. 2).

Stored microsomes

The activity of NAD-cytochrome c oxidoreductase and glucose-6-phosphatase decreased within two weeks and remained nearly unchanged thereafter (fig. 3). The content of cytochrome P 450 and the activity of NADP dependent cytochrome c oxidoreductase remained fairly stable.

Stored liver tissue

Storage of samples of liver tissue had four major effects:

1) The activity of aspartate: 2-oxoglutarate aminotransferase increased 2.5-fold within 3 days. In control experiments the same effect was seen after storage at – 80 °C for 12 h.

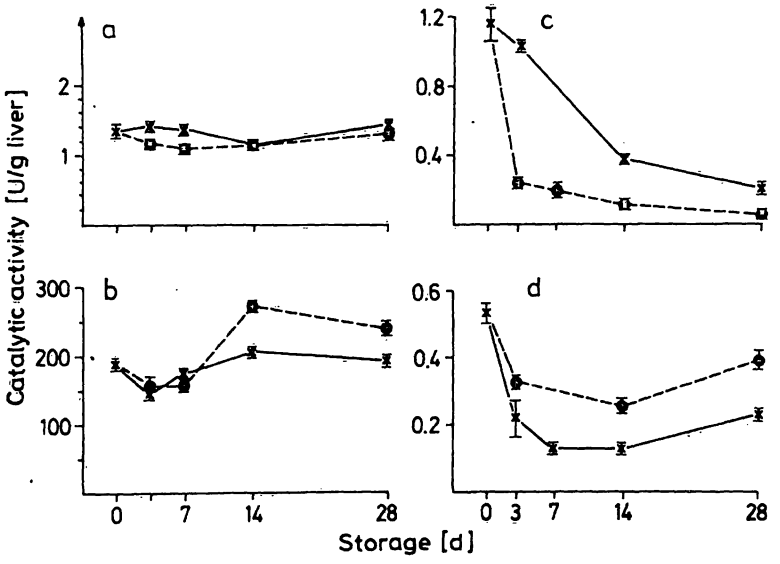

Fig. 1. Effect of storage of supernatant (x—x) or liver tissue (•---•) at – 80 °C on the activities of four cytoplasmic enzymes. Mean values of 4 determinations are given, vertical lines = ± SD. a = *D*-glucose-6-phosphate dehydrogenase; b = arginase; c = glucokinase; d = phosphofructokinase.

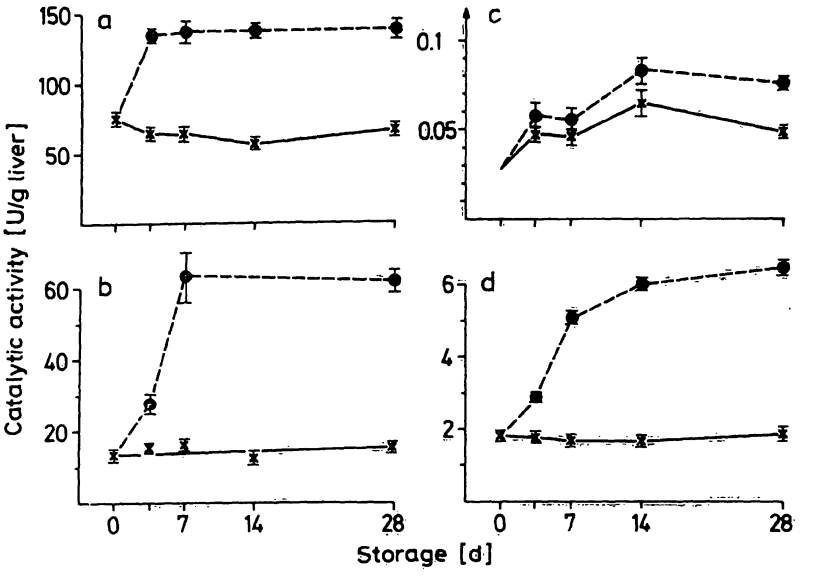

Fig. 2. Effect of storage of supernatant (x—x) or liver tissue (•---•) at – 80 °C on the activities of three (partial) mitochondrial enzymes and xanthine oxidase (c). a = aspartate: 2 oxogluterate aminotransferase; b = glutamate dehydrogenase; d = citrate synthase. For further explanations s. legend to fig. 1.

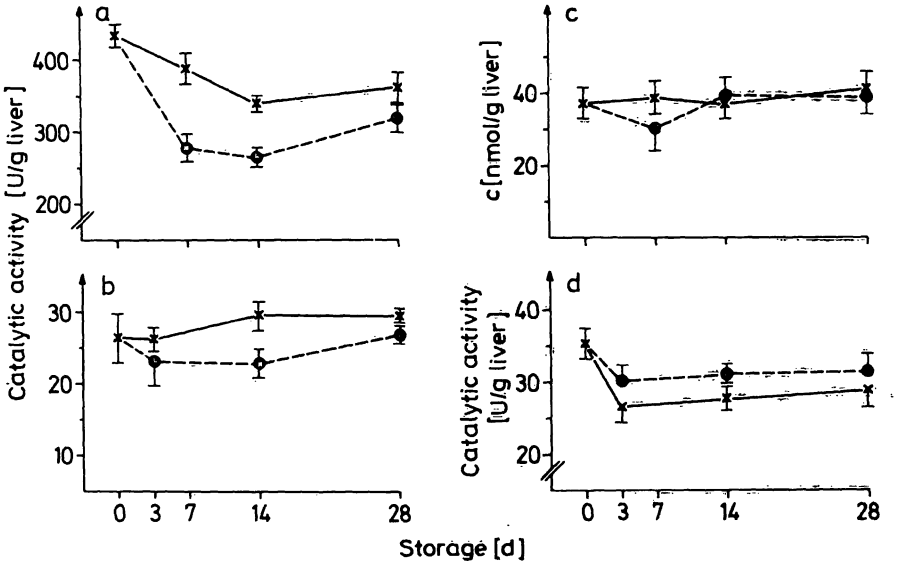

Fig. 3. Effect of storage at – 80 °C on cytochrome P 450 content and microsomal enzyme activities in isolated microsomes (x—x) and liver tissue (•---•). a = NAD specific, b = NADP specific cytochrome c (b 5) oxidoreductase; c = cytochrome P 450 content; d = glucose-6-phosphatase. For further explanations s. legend to fig. 1.

2) An even more pronounced, although less rapid, increase in the activity was seen for glutamate dehydrogenase (fig. 2).

3) A marked increase in the activity was also observed for citrate synthase and xanthine oxidase (fig. 2). However, unlike the changes observed for the two former enzymes, maximal activity was not reached before 2 weeks of storage (fig. 2).

4) The activity of certain other enzymes decreased more markedly in the stored liver samples than in the stored supernatant (fig. 1) or in the stored microsomal pellet (fig. 3).

Discussion

Type of homogenization

The measurement of several enzymes within one sample of liver tissue raises many problems. This is especially true when enzymes of different cellular compartments are to

be determined. The necessity to find a compromise for each particular investigation has repeatedly been documented (3, 4, 9). Our data are in general agreement with earlier reports on the preparation of liver tissue in order to measure enzymes from the cytosol and mitochondrial compartment (3, 4). The main additional information obtained from our study is the pronounced inactivation of ornithine carbamoyl transferase and fructose-biphosphatase by the blade homogenizer even if the latter is used only for a very short period of time. Mechanical factors as well as local increase in temperature, in spite of cooling, due to the high speed of the blade and due to surface tension or bubbles which are regularly produced by this procedure, may contribute to the rapid inactivation of these enzymes. To avoid as far as possible any inactivation of enzymes by the preparation procedure, homogenization with the *Potter-Elvehjem* homogenizer was used for the study of the effects of storage on hepatic enzymes.

Effect of storage

The decrease in activity of glucokinase and of phosphofructokinase during storage at – 80 °C observed in this study is in accordance with the results reported by others for the effect of storage at – 20 °C on glycolytic enzyme activities in liver tissue (5, 6). However, the transitory sharp rise in the activity of phosphofructokinase during the first 3 days of storage reported by *Cryer & Bartley* (6) could not be confirmed. Whether the loss in activity of the glycolytic enzymes is due to the effect of conformational changes or to other factors, like the effect of proteinases (6) or deamidation of amide groups of proteins, which has been shown to occur for the corresponding free amino acids in plasma at – 20 °C (15), remains speculative.

In contrast to earlier findings obtained in storage experiments at – 20 °C (5, 6) the activity of most of the other enzymes remained constant or increased when either liver samples or the supernatant were stored. As already mentioned, the marked increase in the activity of aspartate: 2 oxoglutarate aminotransferase and glutamate dehydrogenase are likely to be due to liberation of these enzymes by disintegration of mitochondria by freezing and thawing. The delayed rise in the activity of glutamate dehydrogenase might be explained by another location of this enzyme within the mitochondria. More difficult to explain is the constant increase in the activity of citrate synthase for two weeks in stored liver tissue and for xanthine oxidase in both liver samples and supernatant. Conformational changes of the enzymes caused by alteration of the water, protein and electrolyte enviroment on freezing and/or extraction may contribute to the increase in the activity of these enzymes.

The results for the effect of storage on the content of cytochrome P 450 in both intact liver or isolated microsomes, are in agreement with earlier findings (13). NAD and NADP-dependent cytochrome c (b 5) oxidoreductase is obviously more stable in isolated microsomes than in the stored intact tissue.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (Bo 373/3).

References

1. Hug, G., Schubert, W. K. & Schwachman, H. (1965) J. Pediat. *67*, 741–749.
2. Neubaur, J. & Hollmann, S. (1966) Klin. Wochenschr. *44*, 723–28.
3. Schmidt, E. & Schmidt, F. W. (1970) Enzymol. Biol. Clin. *11*, 67–129.
4. Schmidt, E. & Schmidt, F. W. (1963) Enzymol. Biol. Clin. *3*, 80–86.
5. Shonk, C. E. & Boxer, G. E. (1964) Cancer Res. *24*, 209–221.
6. Cryer, A. & Co, Bartley, W. (1974) Biochem. J. *144*, 433–434.
7. Kato, R. & Gilette, J. R. (1965) J. Pharmacol. Exp. Ther. *150*, 279–284.
8. Bücher, Th., Luh, B. & Pette, D. (1964) In: Handbuch der physiologisch- und pathologisch-chemischen Analyse (Lang, K., Lehnartz, E., Hoffmann-Ostenhof, O. & Siebert, G. eds.) Bd. VI, p. 292–338, Springer, Berlin.
9. Bergmeyer, H. U. (1974) Methoden der enzymatischen Analyse, Verlag Chemie, Vol. I & II.
10. Wieland, O. (1966) In: Handbuch der physiologisch- und pathologisch-chemischen Analyse (Lang, K., Lehnartz, E., Hoffmann-Ostenhof, O. & Siebert, G. eds.) Bd. VI, B, p. 1–181, Springer, Berlin.
11. Greenberg, D. (1966) In: Handbuch der physiologisch- und pathologisch-chemischen Analyse (Lang, K., Lehnartz, E., Hoffmann-Ostenhof, O. & Siebert, G. eds.) Bd. VI, C, p. 354–367, Springer, Berlin.
12. Mayer, G., Ullrich, V. & Staudinger, H. J. (1968) Hoppe-Seylers Z. Physiol. Chem. *349*, 459.
13. McLean, A. E. M. & Day, P. (1974) Biochem. Pharmacol. *23*, 1173–1180.
14. Bode, Ch., Goebell, H. & Stähler, E. (1968) Z. Klin. Chem. Klin. Biochem. *6*, 418–422.
15. Dickinson, J. C., Rosenblum, H. & Hamilton, P. B. (1965) Pediatrics *36*, 2–13.

Dr. rer. nat. Christiane Bode
Zentrum für Innere Medizin der Universität
Mannkopffstr. 1
D-3550 Marburg/Lahn